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Progress Report on Grant N00014-91-J-1217

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GRANT TITLE: In Vivo Distribution of Liposome Encapsulated Hemoglobin Studied With Imaging Radiotracers

START DATE: 12/01/90

RESEARCH OBJECTIVE: This project has as its objective the development of radiotracer imaging technology to follow the in vivo circulation and organ deposition of liposome encapsulated hemoglobin (LEH). LEH will be labeled with technetium-99m (^{99m}Tc) or indium-111 (^{111}In) and infused into small animals to monitor any in vivo differences between different LEH formulations. These studies will be correlated with any hematological and pathological changes associated with LEH treatment. Development of such non-invasive monitoring techniques may lead to significant cost effective manufacturing and formulation improvements, and ultimately a more efficacious LEH product. The development of this elegant labeling technique should make it possible to study the effect of various LEH modifications on biodistribution non-invasively in primates and humans.

PROGRESS: Our research progress for the period of August 1, 1992 to December 1,1992 is covered in this report. One problem we were asked to address is whether our imaging techniques could distinguish where the various resuscitative fluids go once infused, particularly if they can provide sufficient oxygen to the peripheral tissues. We have designed a series of experiments to help address this issue. Following 50% blood removal from the femoral artery catheter, we infused 2ml of ^{99m}Tc labeled red blood cells and watched the change in their circulation after replacing the additional volume with a resuscitative fluid. To date, we have completed imaging studies for replacement with either normal saline (0.9%), hypertonic saline (7.5% at 5ml/kg), shed blood or no resuscitative fluid. We plan to do the same procedure as with the other resuscitative fluids, but reinfuse LEH when a batch becomes available from NRL within

the next few weeks. We have completed the image analysis for the fluids tested. A region of interest was drawn around various organs and the radioactivity associated with that region compared to the total body activity. Region to total body ratios were determined out to 90 minutes at 15 minute intervals following the infusion of labeled red blood cells. We are most interested in the increases or decreases of blood pool in the peripheral muscles and abdominal organs which are good indicators of the amount of oxygenation of these areas following resuscitation. We saw an increase in activity in the reticuloendothelial system organs of the spleen and liver in the animals receiving no resuscitation or all fluids except red blood cells. There was no significant difference in any of the groups in the heart which represents the blood pool. In the abdomen, there was higher activity for replacement with shed blood and the other fluids over no resuscitation animals. We saw no significant difference in the proximal or distal periphery for any of the groups. Correlation was also made between the trend in mean arterial blood pressure and heart rate following resuscitation for the various fluids. As expected the mean arterial pressure and heart rate of the shed blood group recovered to baseline values after reinfusion, while rats receiving no resuscitative fluid recovered and maintained pressure but heart rate leveled off at 60 beats per minute less than baseline. The saline resuscitations fell between these two control fluids with the hypertonic saline group recovering faster and leveling off, while the 0.9% saline group had a decrease of 20 in mean arterial pressure and a decrease of 50 in heart rate. Although these comparisons have been useful in determining the biodistribution pattern following hypovolemia, we feel this particular imaging technique is not sensitive enough to detect the subtle differences in the resuscitative fluids studied thus far. Yet, these studies have provided some of the first data using imaging technology to follow resuscitation from hypovolemia.

We have completed a series of studies looking at the biodistribution of the ^{99m}Tc labeled LEH infused after a 10% or 50% blood withdrawal in femoral artery cannulated rats. LEH made with bovine hemoglobin and glutathione produced at NRL on July 15, 1992 was used for this study. Unlike the last shipment, this batch had a 94% labeling efficiency with our ^{99m}Tc labeling protocol. Mean arterial pressure, heart rate and temperature were monitored throughout the experiment. Following the bleeding period, ^{99m}Tc labeled LEH was infused via the tail vein. Dynamic images were acquired under the gamma camera for 90 minutes following and at 20 hours post-infusion. At 20 hours, the rats were sacrificed and

tissues counted for radioactivity. These studies will provide the first biodistribution data of LEH given in a rat hypovolemic model. The results with 5 animals in each group showed that there was a significant difference in the circulation persistence and distribution pattern between the two groups. The 50% group maintained approximately 50% of the LEH in the blood pool where it was needed to carry oxygen, while the 10% group had a significant amount of LEH removed from the blood pool by the liver and spleen at 90 minutes. These results were confirmed by both the blood capillaries drawn throughout the experiments, the image data and tissue sampling data. The heart rate and mean arterial pressure did recover following the bleeding period but was slower to recover than previously seen in shed blood resuscitation. As expected the 10% replacement which is equivalent to donating a pint of blood produced only minor changes in mean arterial pressure and heart rate. On the other hand, the 50% replacement rats showed a drop from 97 to 30 in mean arterial pressure which recovered to 91 after 90 minutes following resuscitation with LEH. The heart rate of the 50% replacement rats dropped from 324 to 228 at the end of the bleeding period, but recovered to 336 at 90 minutes following infusion of the LEH. The animals in both groups survived to 20 hours when the autopsies were performed for tissue biodistribution studies.

Since our last report, we have set up a nude mouse tumor model to test the ability of our ^{99m}Tc labeled liposomes to localize to a tumor site. The animals were implanted with 10 million nontransfected Chinese Hamster Ovary cells. After approximately 10 days the tumor-bearing mice were injected with the labeled liposomes and imaged under the gamma camera. We tested both the negative liposomes used for LEH production and our previous infection imaging studies and a neutral liposome formulation comprised of distearoyl phosphatidylcholine:cholesterol: α-tocopherol (2:1:0.02). Both liposome formulations could localize the tumor site. Although we have not analyzed the image data, the tissue data showed a 4 times greater increase in the activity per injected dose for the neutral liposomes as for the negative liposomes.

Also we received a shipment of copper-67 (^{67}Cu) for liposome and red blood cell labeling studies. We tried to label liposomes containing glutathione with ^{67}Cu chelated to the lipophilic carrier pyruvaldehyde methyl thiosemicarbazone (PTSM) and free ^{67}Cu. Although there is evidence in the literature that ^{67}Cu-PTSM labeling may be similar to ^{99m}Tc-HMPAO and interact with glutathione in the body, in our studies the labeling efficiencies have been much lower for the ^{67}Cu-PTSM system than

with the ^{99m}Tc-HMPAO system. We have also used free ^{67}Cu and ^{67}Cu-PTSM to label human and rat red blood cells. The labeling of red cells with copper isotopes such as copper-62 could be used as a positron emission tomography blood pool agent. The human cells labeled at > 90% efficiency while the rat cells labeled at 68% with ^{67}Cu-PTSM and 38% with free ^{67}Cu. The human cells were also more stable following incubation in 50% plasma for 15 hours. The labeled rat red cells were injected into autologous rats and imaged under the gamma camera. The early images for the ^{67}Cu-PTSM rat were very similar to other blood pool images from other red blood cell studies labeled with other isotopes such as ^{99m}Tc.

WORK PLAN: During the next funding period, we will continue to use our technetium-99m labeling protocol to test LEH formulations as supplied by NRL or Vestar for their circulation properties and organ distribution. A LEH formulation being developed by Vestar which can be produced at a smaller more homogeneous size for sterile filtration has been modified for scale up. Biodistribution studies with these new LEH preparations that contain recombinant human hemoglobin will be studied as soon as these preparations become available.

Although our ^{99m}Tc labeling procedure has provided valuable data concerning the biodistribution of LEH, it does not allow us to follow the ultimate metabolic fate of the hemoglobin. This information is very important for the safety of LEH as a blood substitute since we want a product which will be cleared from the body and produce few toxic side effects. To study this problem, we plan to label hemoglobin with ^{3}H and ^{14}C using a mild reductive methylation procedure. This mild labeling technique has been used to label the lysine residues of a number of proteins including hemoglobin without affecting the functionality of the protein. The hemoglobin will be supplied by NRL. Also the labeled starting material used in the procedure is available from commercial sources. Once labeled the hemoglobin will be used to make LEH. The labeled LEH will then be double labeled using the ^{99m}Tc liposome labeling protocol. This double labeled material will be injected into animals and imaged under the gamma camera. The animals will then be sacrificed for tissue biodistribution measurements. Samples of the tissues will be counted for both gamma activity as well as for ^{3}H or ^{14}C using liquid scintillation counting. This study will provide important information concerning the

fate of both the hemoglobin and liposomal components of LEH.

We also plan to study the efficacy of LEH using a positron emitting isotope of oxygen (^{15}O). To our knowledge, this study will be the first attempt to actually quantitate and image oxygen delivery to tissues. These studies are uniquely possible at our institution because of our newly operational cyclotron and positron emission tomography (PET) camera located at our Research Imaging Center and the previous experience of our group in both imaging and blood substitutes. The biodistribution studies using ^{99m}Tc have been very useful, but do not provide any information about how efficient LEH is in delivering oxygen to the tissues in vivo. We will attempt to develop a PET imaging protocol to quantitatively determine the amount of oxygen extracted by the brain, liver and skeletal muscle in rabbits given ^{15}O labeled LEH. The oxygen extraction fraction will be compared to control animals receiving ^{15}O labeled blood. This technique will then be applied to animals subjected to a 50% blood withdrawal and resuscitation.

We also plan to test our ^{99m}Tc labeled liposomes as an imaging agent for the detection of atherosclerotic disease in collaboration with Dr. Bailey at our institution. Such an agent could be used not only to screen patients with the disease, but also to follow the efficacy of cholesterol lowering drugs in the treatment of atherosclerosis.

INVENTIONS: The patent dated October 27, 1992 for ^{99m}Tc labeled liposomes was published and assigned the patent number 5,158,760.

PUBLICATIONS AND REPORTS: We have submitted a manuscript to Critical Care Medicine with Dr. Rudolph at NRL describing our results of the circulation persistence and biodistribution of lyophilized LEH. We plan to submit a manuscript outlining our results using ^{99m}Tc labeled liposomes in infection imaging to Journal of Nuclear Medicine within the next month. We have submitted an abstract entitled " Labeling red blood cells with copper-67" for presentation at the Southwest Chapter of the Society of Nuclear Medicine meeting to be held in Dallas, Texas on March 11-14, 1993. Dr. Phillips was an invited speaker at a symposium entitled "Pharmaceutical Imaging Agents" at the American Association of Pharmaceutical Scientists meeting held in San Antonio on November 15-19, 1992. A progress report was presented at the annual LEH meeting held at NRL, Washington, D.C. on November 6, 1992. The article entitled "Biodistribution Studies of Liposome Encapsulated Hemoglobin (LEH)

Studied With A Newly Developed 99m-Technetium Liposome Label" has been published in Biomaterials, Artificial Cells and Immobilization Technology, Volume 20(2-4), pages 757-760.

TRAINING ACTIVITIES: None